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# Antagonizing activity of vaccinia virus E3L against human interferons in Huh7 cells

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ABSTRACT

The E3L protein of vaccinia virus (VV) is well known for its capacity to evade cellular innate antiviral immunity related to interferon (IFN), for example PKR and RNaseL mediated antiviral activities. However, due to the limited range of cells that support VV E3L deletion mutant replication, the full capacity of E3L inhibiting the innate immune response induced by IFNs remains to be examined. In this report, the inhibition activity of VV E3L against a wide spectrum of human IFNs, including type I IFNs (12 IFN-α subtypes, IFN-β, and IFN-ω), and type II IFN (γ), was comparatively examined using the Copenhagen strain E3L deletion mutant and its revertant control virus in a human hepatoma cell line, Huh7. Deletion of the E3L open reading frame rendered the mutant VV sensitive to all types of IFNs, while the revertant VV was strongly resistant to these treatments. Furthermore, we show that the inhibition of VV E3L deletion mutant by IFN occurs at the stage of intermediate gene translation, while the expression of early genes and transcription of intermediate genes are largely unaffected. Using specific siRNAs to suppress the classical IFN-induced antiviral pathways, we found that PKR is the key factor modulated by E3L, while the RNaseL and MxA pathways play limited roles in this Huh7 cell system. Thus, our data demonstrates that VV E3L can mediate strong inhibition activity against all human type I and type II IFNs, mainly through modulation of the PKR pathway in Huh7 cells.



## Introduction

Interferons (IFNs) play a pivotal role in innate immunity against viral infections. In mammals, IFNs that mediate antiviral activity include: type I IFNs, composed of several IFN-α subtypes, IFN-β, and IFN-ω and a single member of type II IFNs, IFN-γ. The more recent described IFNs-λ1, λ2, and λ3 (alternatively known as IL-29, IL-28A/B), are induced by viral infections similar to type I IFNs, and have been shown to exhibit antiviral and antiproliferative activities *in vitro* and in response to poxvirus infection *in vivo* (Meager et al., 2005; Onoguchi et al., 2007; Bartlett et al., 2005). Both type I and type II IFNs induce the JAK/STAT signaling pathway in all nucleated cell types and transcriptionally regulate several interferon stimulated genes (ISGs) that result in the production of antiviral proteins, including PKR, 2′–5′ OAS, and myxovirus resistance protein (MxA). All IFN-α subtypes are similar in structure and share a common receptor (IFNAR1/2), and can elicit antiviral activity, but may also vary in their ability to activate signaling pathways in certain cell types, and are likely to induce different genes (Foster and Finter, 1998; Pestka et al., 2004; Pfeffer, 1997; Yano et al., 2006). Thus, it is entirely possible that different IFN subtypes may initiate different degrees of antiviral activity.

Millions of years of virus and host co-evolution have led viruses to develop a wide variety of strategies to antagonize IFN activity. Of interest, a dsRNA binding protein encoded by vaccinia virus (VV), E3L, is well known for its capacity to block IFN induction, signaling, and activity. IFN inducible dsRNA-dependent Protein Kinase R (PKR) and 2′–5′-oligoadenylate synthetase (2′–5′ OAS) are activated in the presence of dsRNA which leads to the inhibition of translation initiation through eIF2α phosphorylation and the degradation of mRNA by ribonuclease RNaseL, respectively (Garcia et al., 2006; Carroll et al., 1997). Competitive binding of E3L to dsRNA inhibits the activation and overall function of both PKR and 2′–5′ OAS and has been associated with the inhibition of IRF-3 phosphorylation and the suppression of IFN-β induction (Chang et al., 1992; Davies et al., 1993; Xiang et al., 2002; Smith et al., 2001). The similar strategy to antagonize IFN antiviral function through dsRNA binding is also used by several other vertebrate viruses such as reovirus σ3, HSV-1 US11, avian reovirus σA, and influenza virus NS1 (Imani and Jacobs, 1988; Jacobs and Langland, 1998; Langland et al., 2006; Lu et al., 1995).

Deletion of E3L (VVΔE3L) is associated with a highly restricted host range. For example, in comparison with the wild-type virus, VVΔE3L is unable to replicate in Vero, murine L929, or HeLa cells (Beattie et al., 1996; Langland and Jacobs, 2002; Shors et al., 1998, 1997). It is well known that PKR and RNaseL, both of which can be regulated by IFNs, play major roles in determining the highly restricted host range of a VV E3L deletion mutant (Langland and Jacobs, 2002; Beattie et al., 1996; Xiang et al., 2002). However, the interplay between E3L and IFN-induced cellular responses (which involves more than 300 genes) in

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relation to virus replication has not been well characterized due to the limited cell lines available for such analysis. Previous studies on IFN sensitivity of a VV E3L deletion mutant were based on HeLa (Langland and Jacobs, 2004) or L929 cells (Beattie et al., 1995a,b; Xiang et al., 2002). However, in these cells, the replication of a VV E3L deletion mutant is defective. A VV E3L deletion mutant can replicate in rabbit kidney, RK-13 cells (Chang et al., 1995), and was shown to be sensitive to treatment with rabbit IFN-α in this cell line (Shors et al., 1997). Nonetheless, it is unlikely that the true capacity of E3L inhibiting human IFN antiviral activity can be analyzed using these cell lines and the interplay between E3L and IFN-induced antiviral signaling remains unclear.

In this study, we found that the VV E3L deletion mutant can efficiently replicate in Huh7 cells. Using this cell line, we show that E3L can mediate potent inhibition on antiviral activity induced by type I and type II IFNs through modulation of the PKR pathway.

## Results

### *The VV E3L deletion mutant can replicate efficiently in the human hepatoma cell line Huh7*

The E3L deletion mutant (Cop-ΔE3L) and its revertant (Cop-ΔE3L-Rev) were constructed based on the Copenhagen strain as described in Materials and methods. The only difference between the E3L deletion mutant and revertant viruses is the absence and presence of the *E3L* ORF, respectively (Fig. 1a). We confirmed the absence and presence of the E3L protein in the E3L deletion mutant and revertant viruses by Western blot (data not shown). The difference between the revertant virus and the wild-type Copenhagen strain is the inclusion of gpt (for positive selection) and EGFP for easy plaque identification and orientation of the E3L ORF. The inclusion of gpt and EGFP has no effect on virus replication and IFN sensitivity (data not shown). As shown in Fig. 1a, the mRNA expression of two adjacent ORFs, *E2L* and *E4L*, was not affected by deletion of *E3L*. The replication capability of the E3L deletion mutant was tested in six human cell lines and compared to BHK21. The human hepatoma cell line Huh7, was the only cell line to efficiently support replication of the E3L deletion mutant, while the E3L deletion mutant completely failed to replicate in MRC5, AD293, and HeLa cells (Fig. 1b).

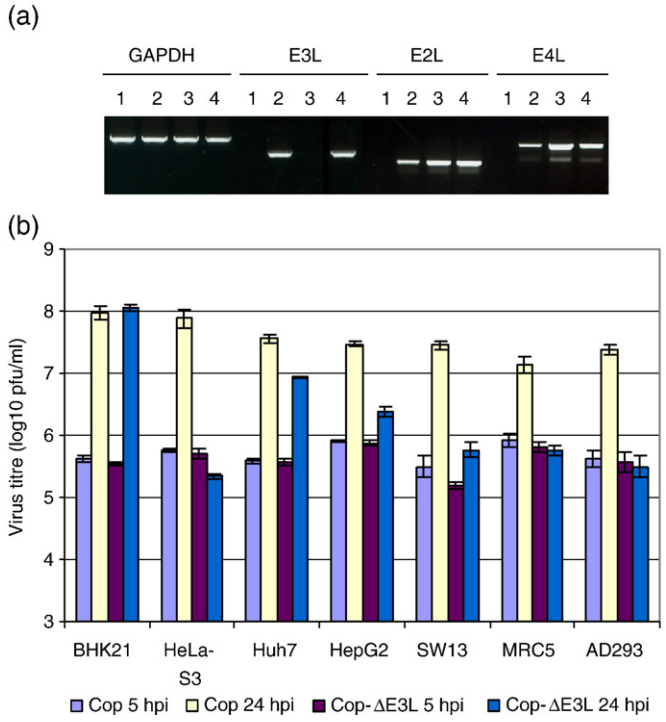


**Fig. 1.** Confirmation of the deletion of VV E3L in the E3L deletion mutant and the expression of E3L in the revertant control viruses. (a) Total RNA was extracted from BHK21 cells infected with Cop-ΔE3L, Cop-ΔE3L-Rev, and wild-type VV Cop. RT-PCR analysis confirm *E3L* mRNA expression is absent in the BHK21 cell control (lane 1), and in the E3L deletion mutant (Cop-ΔE3L) (lane 3), but is present in wild-type VV Cop (lane 2) and in the revertant control (Cop-ΔE3L-Rev) (lane 4). Deletion of *E3L* did not affect the mRNA expression of adjacent ORFs, *E2L* and *E4L*. (b) Human hepatocellular carcinoma, Huh7 cells, efficiently support Cop-ΔE3L replication. Confluent cell monolayers were infected at a MOI of 1 with wild-type VV Copenhagen strain (Cop) and Cop-ΔE3L and harvested 5 and 24 hpi. Virus titres ($\log_{10}$ pfu/ml) were measured in triplicate. Error bars indicate standard error of the mean.

### *VV E3L is a potent inhibitor to all human type I and type II interferons in Huh7 cells*

Huh7 cells have been reported to be responsive to human type I IFNs (Blight et al., 2002). We confirmed the Huh7 cell line we used in this study is responsive to both type I and type II IFNs by activation of STAT1 by phosphorylation at two residues, tyrosine 701 and serine 727 (Supplementary Fig. 1).

To analyze the inhibition activity of the E3L protein on human IFNs in Huh7 cells, Cop-ΔE3L and its revertant were comparatively analyzed for their sensitivity to both type I and type II IFNs (including the 12 IFN-α subtypes, IFN-β, IFN-ω, and IFN-γ) (Fig. 2). As shown in Fig. 2a, Cop-ΔE3L-Rev is resistant to treatment of all type I and type II IFNs tested, depicted by the consistent pattern of replication of the revertant virus in the presence of all IFN species. Generally, a slight decrease in Cop-ΔE3L-Rev replication is seen with an IFN dose of 200 to 2000U/ml of type I and type II IFN species.

In contrast, deletion of the *E3L* gene results in a dramatic increased sensitivity to all human IFN species: IFN-β, IFN-ω, IFN-γ, and the IFN-α subtypes. As illustrated in Fig. 2b, replication of Cop-ΔE3L is inhibited in the presence of type I and type II IFNs in a dose-dependent manner. Even at a low dose of 20U/ml, IFN species demonstrated potent inhibition, decreasing virus replication by 100-fold, seen in IFN-β, IFN-ω, and IFN-γ treated Huh7 cells. Among all IFNs tested, the most potent IFN species was IFN-α14, which inhibited replication of Cop-ΔE3L by 1000-fold at its highest dose of 2000U/ml. Following IFN-α14, were type I IFNs: IFN-α2a, IFN-β, and type II IFN-γ, each of which inhibited replication of Cop-ΔE3L between 100 to 1000-fold. In contrast, the least effective at inhibiting viral replication was IFN-α7, followed by IFN-α5.

### *Cop-ΔE3L IFN sensitivity is mediated by PKR*

To further characterize the mechanism of IFN resistance of Cop-ΔE3L-Rev and sensitivity of Cop-ΔE3L mutant viruses (Fig. 2), we examined the roles of each of the three main IFN-induced classical antiviral pathways: PKR, 2′–5′ OAS/RNaseL, and MxA pathways. Since IFNs transcriptionally induce PKR, to investigate the PKR pathway, we first monitored any change in endogenous PKR protein levels in type I (leukocyte, -β,-ω) and type II (γ) IFN-treated Huh7 cells in the absence and presence of virus infection with Cop-ΔE3L-Rev and Cop-ΔE3L. As shown in Fig. 3a, the level of PKR in Cop-ΔE3L-Rev infected Huh7 cells is comparable to the level of PKR in the cell control in the absence of IFN treatment. However, the level of total PKR appears to be reduced in Huh7 cells infected with Cop-ΔE3L in the absence of IFN treatment. Treatment with type I (leukocyte, -β,-ω) and type II (γ) IFNs slightly enhanced the level of endogenous PKR in the absence of virus infection. Even in the presence of both type I and type II IFNs, total PKR seems to be slightly reduced in all Cop-ΔE3L infected cells, whereas in all Cop-ΔE3L-Rev infected cells, there is no difference in PKR in the absence or presence of IFN treatment. It is important to note that the antibody used in this study recognizes endogeneous PKR protein and was

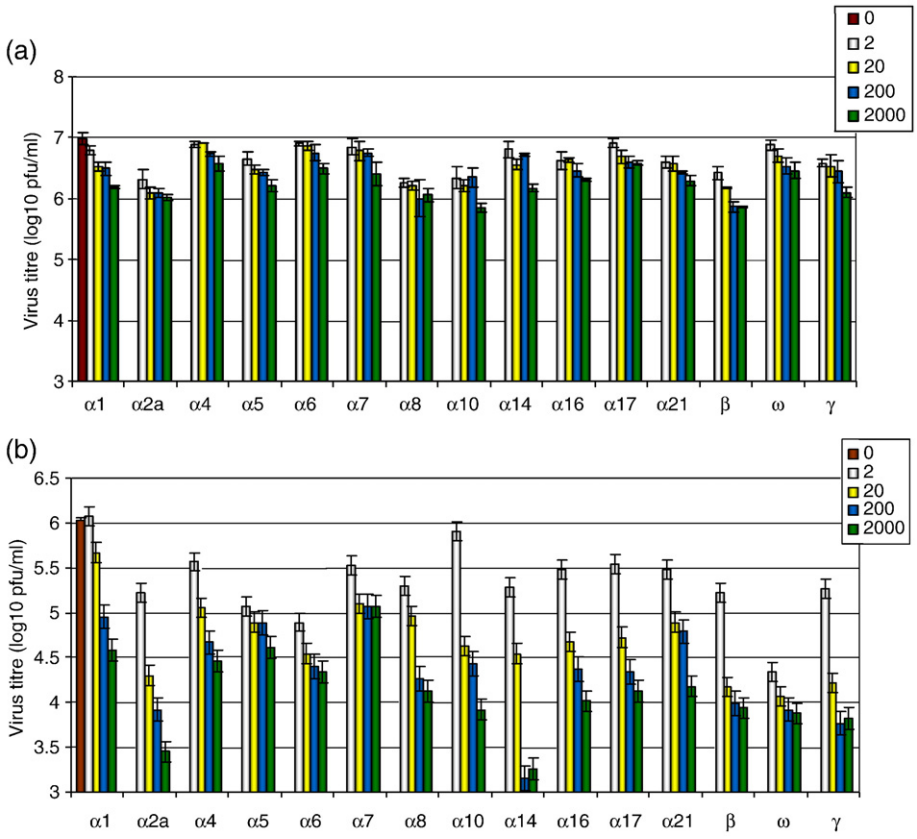


**Fig. 2.** Comparative analysis of the sensitivity of Cop-ΔE3L-Rev and Cop-ΔE3L to type I and type II human IFNs. (a) Replication of Cop-ΔE3L-Rev is resistant to IFN treatment. (b) Replication of Cop-ΔE3L is inhibited by type I and type II human IFNs in a dose-dependent manner. Huh7 cell monolayers ($5\times10^5$ cells/ml) were pretreated with human IFNs (12 IFN-α subtypes, IFN-β, ω, and γ) at different concentrations of 0, 2, 20, 200, or 2000 U/ml for 24 h at 37 °C. The IFN-treated cells were infected at a MOI of 1, with Cop-ΔE3L-Rev or Cop-ΔE3L and harvested 24 h later. Titrations were performed in triplicate. Error bars indicate standard error of the mean.

synthesized against residues adjacent to a critical phosphorylation residue, Thr451. Thus, because of this antibody's specificity at detecting endogeneous PKR, the absolute total PKR protein level may not be fully detected as PKR dimerizes and autophosphorylates following virus infection. We next examined the activation/phosphorylation of PKR and corresponding phosphorylation changes in its substrate, eIF2α. Fig. 3a demonstrates phosphorylated PKR is in abundance following infection with Cop-ΔE3L and is enhanced after IFN treatment. A minimal level of phosphorylated PKR is present in Huh7 cells infected with Cop-ΔE3L-Rev in the absence and presence of IFN treatment. Total eIF2α levels slightly increased post viral infection, regardless of IFN treatment. The level of phosphorylated eIF2α increases after viral infection and IFN treatment. The only exception is observed in IFN-β treated Huh7 cells, in which total eIF2α and phosphorylated eIF2α is slightly reduced in the absence of virus infection (Fig. 3a). Although infection with both Cop-ΔE3L-Rev and Cop-ΔE3L induced eIF2α phosphorylation, a significantly greater level of phosphorylated eIF2α is present in Huh7 cells infected with Cop-ΔE3L than Cop-ΔE3L-Rev.

To further characterize the role of the PKR pathway in the IFN sensitivity of Cop-ΔE3L, we used siRNA to specifically suppress the expression of PKR in Huh7 cells. Huh7 cells transfected with PKR siRNA were treated with 100U/ml of leukocyte IFN, IFN-β, IFN-ω, and IFN-γ and infected with Cop-ΔE3L for 24h. In all cells transfected with PKR siRNA (Fig. 3b), the expression of PKR was significantly reduced. Suppression of PKR in Huh7 cells abolished the effect of type I and type II IFN antiviral activity, in that when transfected with PKR siRNA, the replication of Cop-ΔE3L in the presence of type I and type II IFNs was at the same level as or higher than the control without IFNs (Fig. 3c). In addition, in comparison with the control cells not transfected or transfected with the negative siRNA, the knockdown of PKR expression significantly enhanced the replication of Cop-ΔE3L in Huh7 cells not treated with IFN.

It is intriguing that transfection with the negative siRNA also moderately enhanced Cop-ΔE3L replication. To verify that transfection of Huh7 cells with this negative siRNA does not affect the antiviral activity of type I and type II IFNs, Huh7 cells were also transfected with the negative control siRNA then subsequently treated with 100U/ml of leukocyte IFN, IFN-β, IFN-ω, and IFN-γ and infected with Cop-ΔE3L. The E3L deletion mutant remains sensitive to IFN treatment, indicating that the increase in virus titre in all negative control siRNA transfected cells in the absence of IFN treatment is an effect specific to this particular control siRNA, regardless of Cop-ΔE3L IFN sensitivity to type I and type II IFN (Fig. 3c).

### *RNaseL is not expressed in the Huh7 cells*

To examine the role of the 2′–5′ OAS/RNaseL pathway in the cellular response to Cop-ΔE3L infection, we monitored RNaseL activity by measuring 28S and 18S rRNA degradation in IFN-treated and VV infected Huh7 cells. As depicted in Fig. 4a, RNaseL activity was not detected in Huh7 cells observed at 6 and 12hpi. Given the limited RNaseL activity in this cell system, we monitored the endogeneous RNaseL protein in Huh7 cells and in a positive control, HeLa-S3 cells, by Western blot. Total RNaseL protein was undetectable in Huh7 cells even in the presence IFN treatment and virus infection, yet present in HeLa-S3 cells (Fig. 4b). To further examine the endogenous expression of *RNaseL* mRNA, we performed RT-PCR analysis of RNA extracted from Huh7 cells treated with type I and type II IFNs and infected with both Cop-ΔE3L and Cop-ΔE3L-Rev. As shown in Fig. 4c, *RNaseL* mRNA is detected in HeLa-S3 cells, while it is not detected in Huh7 cells even in the presence of IFN treatment and virus infection. Since we were

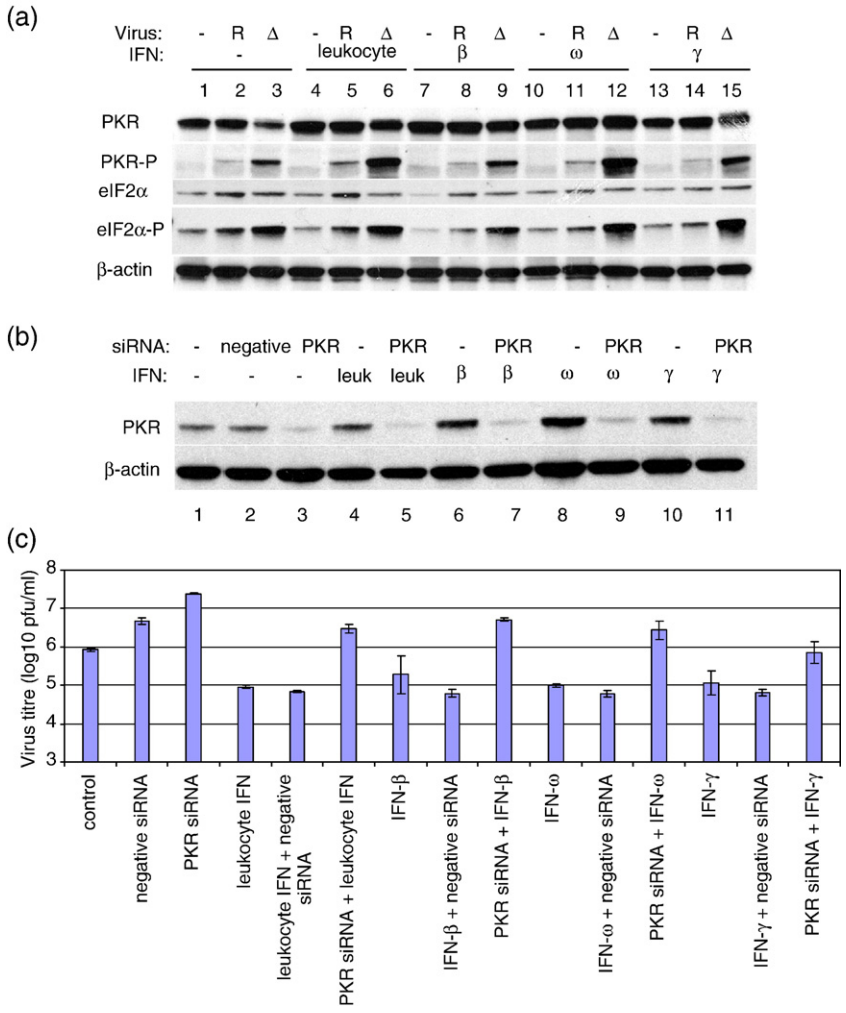


**Fig. 3.** E3L evades the PKR/eIF2α pathway in Huh7 cells. (a) Western blot analysis of endogenous and phosphorylated levels of PKR and eIF2α in Huh7 cells with no IFN (lanes 1, 2, and 3) or treated with leukocyte IFN (lanes 4, 5, and 6), IFN-β (lanes 7, 8, and 9), IFN-ω (lanes 10, 11, and 12), IFN-γ (lanes 13, 14, and 15), and infected with Cop-ΔE3L-Rev (R) or Cop-ΔE3L (Δ). (b) Western blot confirms the knockdown of PKR protein levels in Huh7 cells transfected with PKR siRNA in the absence (lane 3) and presence of leukocyte IFN (lane 5), IFN-β (lane 7), IFN-ω (lane 9), and IFN-γ (lane 11). Controls include Huh7 cells only (lane 1), and negative siRNA transfection (lane 2). (c) Knockdown of the PKR pathway by siRNA enhances Cop-ΔE3L replication in the absence and presence of type I and type II IFNs in Huh7 cells. Negative siRNA transfections do not affect Cop-ΔE3L IFN sensitivity. Huh7 cells were untreated (control) and transfected with negative siRNA (negative), PKR siRNA, and treated with leukocyte IFN, IFN-β, IFN-ω, and IFN-γ. PKR and negative siRNA transfected cells and IFN treatment are as indicated. Titrations were performed in triplicate. Error bars indicate standard error of the mean.

unable to detect the basal level of RNaseL in Huh7 cells by Western blot and RT-PCR, RNaseL does not contribute to the IFN sensitivity of Cop-ΔE3L in Huh7 cells.

### *IFN-induced MxA does not inhibit Cop-ΔE3L replication*

Although the antiviral mechanism of MxA remains unknown, IFN-induced MxA expression has been linked to the inhibition of virus gene transcription, viral mRNA translation, and virus nucleocapsids transportation of several RNA viruses (Hefti et al., 1999). Currently, no evidence has been reported of the role of MxA in the cellular immune response to poxviruses. To examine the inhibitory potential of MxA activity with regards to Cop-ΔE3L replication, we used MxA specific siRNA to knockdown the expression of MxA and observed this effect on virus replication. The knockdown of MxA expression in Huh7 cells transfected with MxA siRNA and treated with leukocyte IFN, IFN-β, IFN-ω, and IFN-γ was confirmed by RT-PCR (Fig. 5a). Type I IFNs (leukocyte IFN, IFN-β, and IFN-ω) induced stronger MxA expression than IFN-γ. MxA expression was not detected in cells not treated with IFN. Knockdown of MxA in Huh7 by siRNA did not significantly affect the ability of the E3L deletion mutant to replicate in the absence or presence of leukocyte IFN, IFN-β, IFN-ω, or IFN-γ (Fig. 5b). Cop-ΔE3L remained sensitive to type I and type II IFN treatment in Huh7 cells transfected with MxA siRNA.

### *IFNs inhibit translation of intermediate genes in Cop-ΔE3L infections*

As we have observed a stronger level of eIF2α phosphorylation in Cop-ΔE3L than in Cop-ΔE3L-Rev infected Huh7 cells (Fig. 3a), we next investigated the association of such eIF2α phosphorylation with virus protein synthesis. We measured the effect of IFN treatment on the expression of two VV genes, an early gene, *D12L*, which encodes for a capping enzyme (Higman et al., 1994), and an intermediate gene, *G8R*, whose protein product has been identified as a late transcription factor (Keck et al., 1990). RT-PCR analysis demonstrate the mRNA expression of *D12L* and *G8R* in Huh7 cells infected with both the revertant control and E3L deletion mutant viruses was not impaired in either the absence or presence of type I and type II IFNs (Fig. 6a). However, at the level of protein expression, translation of the early gene, *D12L*, was largely unaffected in either the absence or presence of IFN or virus infection. In contrast, type I IFN treatment completely blocked translation of G8R, while type II IFN treatment also significantly inhibited G8R protein synthesis. It should be noted that G8R protein translation was compromised in Cop-ΔE3L infections in

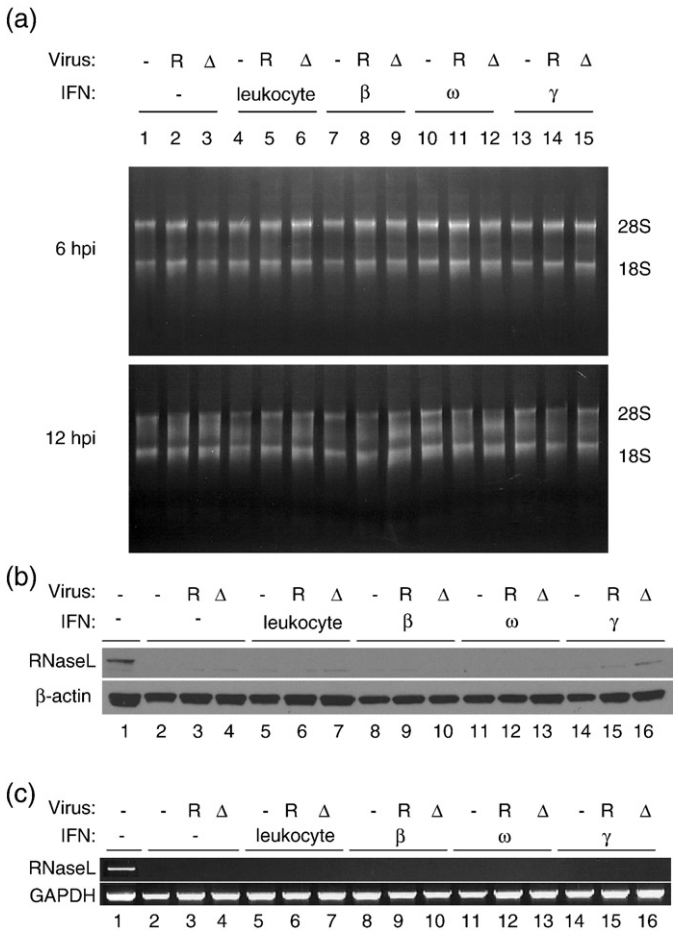


**Fig. 4.** (a) No RNaseL activity is present in Huh7 cells treated with type I and II IFNs and infected with Cop-ΔE3L-Rev (R) and Cop-ΔE3L (Δ). 18S and 28S ribosomal subunits remain intact 6 and 12 hpi as indicated. (b) RNaseL protein is detected in HeLa-S3 cells (lane 1) and is absent in Huh7 cells in the absence of IFN treatment and virus infection (lane 2) and in the presence of leukocyte IFN (lanes 5, 6, and 7), IFN-β (lanes 8, 9, 10), IFN-ω (lanes 11, 12, 13), and IFN-γ (lanes 14, 15, and 16) and virus infection with Cop-ΔE3L-Rev (R) and Cop-ΔE3L (Δ). (c) *RNaseL* mRNA expression is present in HeLa-S3 cells (lane 1), but is undetectable in Huh7 cells untreated or treated with type I and type II IFNs and infected with Cop-ΔE3L-Rev (R) and Cop-ΔE3L (Δ) as indicated.

comparison with Cop-ΔE3L-Rev infections even in the absence of IFN treatment (Fig. 6b). This partial block of G8R may account for the difference in the replication capacity between Cop-ΔE3L and Cop-ΔE3L-Rev in Huh7 cells (Fig. 1b).

## Discussion

IFNs can regulate the expression of over 300 genes (de Veer et al., 2001). PKR and RNaseL pathways are the two best characterized innate antiviral immune responses regulated by IFNs. It is well known that vaccinia E3L, a double-stranded RNA binding protein, plays a critical role in determining the virus tropism by inhibiting activation of PKR and RNaseL (Langland and Jacobs, 2004; Beattie et al., 1996; Xiang et al., 2002). IFN-induced antiviral activities, including activation of PKR and RNaseL, against vaccinia virus were examined in HeLa (Langland and Jacobs, 2004) or L929 (Beattie et al., 1995b) cells using a wild-type vaccinia virus and its E3L deletion mutant. However, the replication of the E3L deletion mutant virus is defective in these cell lines even without IFN treatment. By co-expression of E3L, IFN-induced antiviral activities can be compromised against VSV replication (Shors et al., 1998). A rabbit kidney cell line, RK13, can efficiently support Cop-ΔE3L replication and this replication could be blocked by rabbit IFN-α (Chang et al., 1995). However, due to the limited availability of reagents, E3L mediated IFN resistance particularly against human IFNs was not comprehensively examined. In this report, we describe a human hepatoma cell line Huh7, which is IFN responsive and can efficiently support the replication of a vaccinia E3L deletion mutant. The capacity of vaccinia E3L suppressing both human type I (12 IFN-α subtypes, IFN-β, and IFN-ω) and type II (IFN-γ) IFN-induced antiviral activities against vaccinia virus was investigated using this cell line. In this study, we demonstrated that VV E3L plays the major role in mediating antagonistic activity against all human type I and type II IFNs in a human liver carcinoma cell line, Huh7. To our knowledge, we provide the first comprehensive analysis of the inhibitory activity of E3L against all human type I and type II IFNs in a human cell line. In this report, we made three contributions to characterizing the IFN antagonizing properties of VV E3L. First, we identify a human cell line, Huh7 cells, which can efficiently support the replication of the VV E3L deletion mutant and is suitable to further examine the antiviral activity of a wide range of human IFNs and/or viral IFN antagonists. Second, in this Huh7 cell system, we conclusively demonstrated that VV E3L is a universal antagonist against both type I and type II IFNs. Third, in this specific virus/cell system, PKR is the major IFN regulated signal suppressed by VV E3L.

In humans, several type I IFN species: 12 IFN-α subtypes, IFN-β, IFN-ω, and one type II IFN-γ have been identified. Interferon-regulatory factor 3 (IRF-3), mediates the first wave of IFN-α4 and IFN-β production, and is followed by the expression of the latent transcription factor, interferon regulatory factor 7 (IRF-7), which mediates the second wave of IFN production, inducing expression of *IFN-α2*, *α5*, *α6*, and *α8* (Pestka et al., 2004; Levy et al., 2003; Sato et al., 2000; Decker et al., 2002). The difference in induction times and variable biological functions associated with the 12 IFN-α subtypes make it possible for variable degrees of antiviral strength. We show that different IFN-α subtypes exhibit different levels of inhibition activity against the E3L deletion mutant. The specific mechanisms underlying this variation in inhibition activity among IFN-α subtypes and type II IFN remain to be investigated.

The E3L deletion mutant induced a greater degree of eIF2α phosphorylation than infection with the revertant virus in both the absence and presence of IFN treatment (Fig. 3a). The increased level of eIF2α phosphorylation associated with Cop-ΔE3L infection may play a critical role in IFN-induced inhibition of the virus replication, since it has been shown that a 10–20% difference of eIF2α phosphorylation could have significant impact on the inhibition of protein translation (Hinnebusch, 2000).

In Cop-ΔE3L-Rev infected Huh7 cells, phosphorylated eIF2α was present although a low level of phosphorylated PKR was detected (Fig. 3a). In addition to PKR, three other cellular kinases have been shown to phosphorylate eIF2α in regulating global translation in response to distinct stimuli. These kinases include GCN2, triggered by amino acid starvation; PERK, activated by cellular stress and protein misfolding at the ER membrane; and HRI, which is responsive to iron deficiency (He, 2006). It is possible that these additional kinases may contribute to eIF2α phosphorylation in Cop-ΔE3L-Rev infected cells, although the actual kinase involved in this case is unknown. In the system described in this study, the level of phosphorylated PKR coincides with the enhanced level of eIF2α phosphorylation in Cop-ΔE3L infected Huh7 cells (Fig. 3a). This is in agreement with several previous studies, which have shown that an increased level of eIF2α phosphorylation induced by Cop-ΔE3L infection is associated with an increased level of PKR/phosphorylated PKR (Chang et al., 1992; Sharp et al., 1998; Seet et al., 2003). In the present study, we are the first to show this association in the context of IFN treatment and virus infection. In addition, we clearly show that specific suppression of PKR level by siRNA in Huh7 cells rescues Cop-ΔE3L replication upon type I and type II IFN treatment. Apart from its dsRNA binding capability and the subsequent inhibition of PKR activation, the E3L protein has also been shown to directly bind to both regulatory and substrate binding regions of PKR and inhibit PKR function (Sharp et al., 1998). Thus, our data indicates that the E3L protein exerts its antagonizing activity against

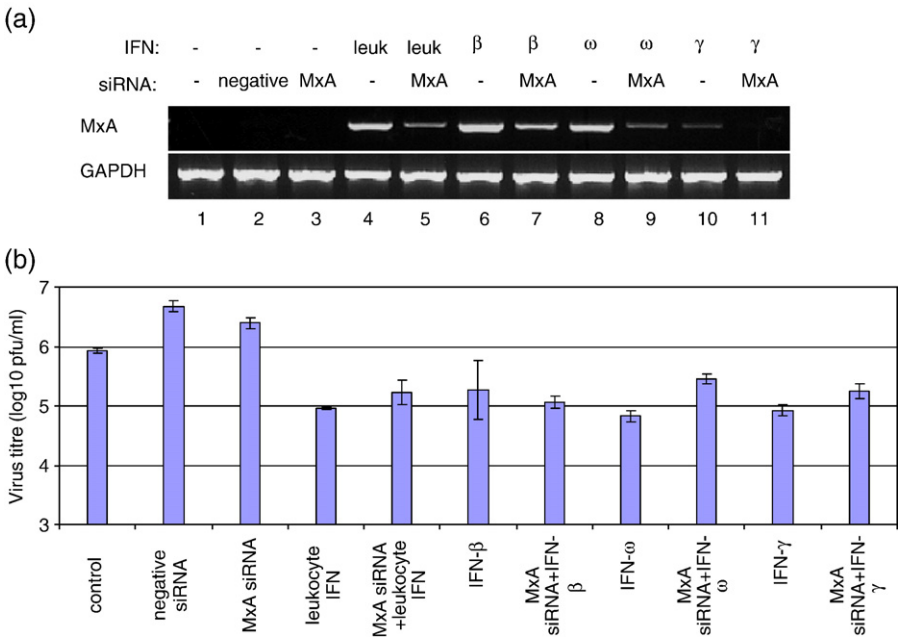


**Fig. 5.** Effect of MxA knockdown by siRNA on the replication of Cop-ΔE3L in Huh7 cells in the absence and presence of type I and type II IFNs. (a) Knockdown of type I and type II IFN-induced MxA expression in Huh7 cells by siRNA, confirmed by RT-PCR. (b) Effect of MxA activity on Cop-ΔE3L replication. Huh7 cells transfected with MxA siRNA and treated with type I and type II IFNs are as indicated. Virus control in Huh7 cells only (control) and negative siRNA transfection control (negative siRNA) are included. Titrations were performed in triplicate. Error bars indicate standard error of the mean.

IFN by blocking PKR function to activate antiviral signals, such as eIF2α phosphorylation, in the cell system described here. In addition to its classical role in phosphorylating eIF2α, PKR is also implicated in the regulation of other signaling pathways such as proinflammatory cytokines, growth factors, oxidative stress and apoptosis (Garcia et al., 2006) and such activities may also contribute to the IFN-induced inhibition of Cop-ΔE3L replication in Huh7 cells described in this report and pathogenesis *in vivo*.

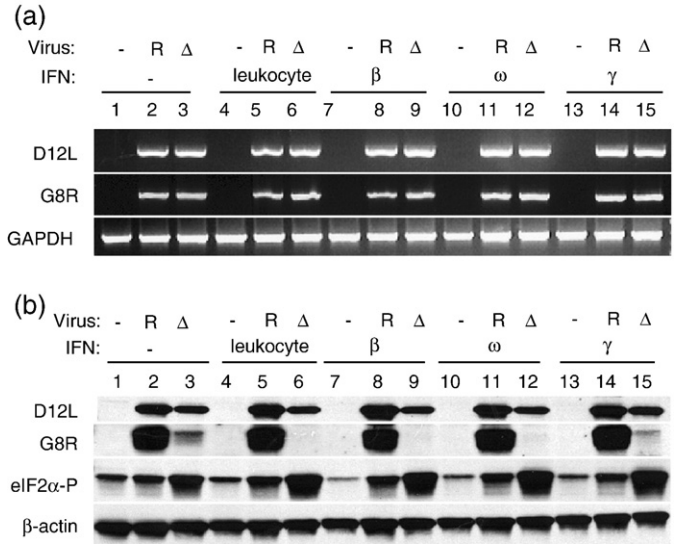


**Fig. 6.** IFN treatment inhibits VV intermediate protein synthesis. (a) Transcription of VV early gene, *D12L*, and intermediate gene, *G8R*, is not affected by IFN treatment in Huh7 cells infected with Cop-ΔE3L-Rev (R) or Cop-ΔE3L (Δ). Total RNA was extracted from Huh7 cells treated with 1000 U/ml of leukocyte IFN, IFN-β, IFN-ω, and IFN-γ for 24 h at 37 °C and infected with Cop-ΔE3L-Rev (R) and Cop-ΔE3L (Δ) at a MOI of 5 for 6 h. *D12L*, *G8R* and human *GAPDH* as a control were amplified by RT-PCR. (b) Absence of the G8R protein in all Cop-ΔE3L mutant infected Huh7 cells treated with type I and type II IFNs. Western blot analysis of Huh7 cells treated with type I and type II IFNs (1000 U/ml) and infected with Cop-ΔE3L-Rev (R) and Cop-ΔE3L (Δ) at a MOI of 5 for 6 h, using polyclonal antibodies against D12L and G8R.

A recent study has shown that PKR is the key factor in determining the replication defective phenotype of the VV E3L deletion mutant in HeLa cells (Zhang et al., 2008). It requires at least 12 h of incubation (data not shown) for IFNs to mount effective antiviral signaling, while replication of the VV E3L deletion mutant is aborted at early times of infection (Ludwig et al., 2006; Chang et al., 1992). Therefore, it is unlikely that PKR mediated antiviral activity, which is antagonized by the E3L protein, in HeLa cells is regulated by IFN. Here, we present strong evidence showing that the E3L protein antagonizes antiviral signaling pathways induced by IFN, in which PKR is the main player. Since IFNs can regulate the expression level of greater than 300 genes simultaneously (de Veer et al., 2001), it is very likely that the mechanism of IFN-induced and PKR mediated antiviral signaling observed in Huh7 cells is different to the recent report on the role of PKR in restricting the replication of the vaccinia E3L deletion mutant in HeLa cells (Zhang et al., 2008).

Evasion of the 2′–5′ OAS pathway by E3L was shown in various cell systems that include PKR knockout murine fibroblasts and immortalized MEFs whose constitutive RNaseL expression suppressed VVΔE3L, but had no effect on wild-type VV (Rivas et al., 1998; Xiang et al., 2002). More recently, the E3L protein of modified vaccinia virus (MVA) was shown to inhibit the 2′–5′ OAS/RNaseL pathway in HeLa cells (Ludwig et al., 2005). Although the 2′–5′ OAS/RNAseL pathway is induced by both type I and type II IFNs, the induction of antiviral pathways may be cell type-specific (Stark et al., 1998). In this study, RNaseL activity was not detectable in Huh7 cells treated with type I and type II IFNs during VV infection. We further demonstrate the lack of RNaseL mRNA and protein expression in this cell line. Therefore, the role of E3L inhibiting RNaseL mediated antiviral activity cannot be evaluated using Huh7 cells as described in this report.

However, it is plausible to further investigate the role of E3L at inhibiting RNaseL activity using this human cell line. To elucidate the function of RNaseL in inhibiting replication of the E3L deletion mutant, we can generate constitutively expressing Huh7-RNaseL cell lines. This would allow for an expanded characterization of VV E3L and its inhibition of RNaseL activity in human cells.

MxA is induced primarily by type I IFNs or type III IFNs (Haller et al., 2007) and has been reported to inhibit both RNA and DNA viruses (Stark et al., 1998; Gordien et al., 2001; Bartlett et al., 2005). The

mechanism by which MxA inhibits viral replication remains unknown and the role of this IFN-induced protein in suppressing vaccinia virus replication has not been elucidated. In this study, we found that MxA has limited activity in suppressing Cop-ΔE3L replication. Knockdown of MxA gene expression by siRNA does not significantly abolish the antiviral activity of either type I or type II IFNs against Cop-ΔE3L. It is also possible that MxA mediated antiviral signaling is defective in Huh7 cells, as this antiviral activity has been shown to be cell type-specific (Schneider-Schaulies et al., 1994; Landis et al., 1998).

VV replication was shown to be sensitive to pretreatment with murine IFNs-α/β in Balb/c mice and that this inhibition is associated with the blockage of early viral gene expression (Rodriguez et al., 1991). In the human cell line, Huh7, we show that both type I and type II IFNs inhibit intermediate ORFs translation of a VV mutant devoid of the E3L ORF, while early and intermediate gene transcription is unaffected. IFNs exert a different spectrum of antiviral activity through synchronizing with other cellular factors such as TNF-α *in vivo* in comparison with the *in vitro* tissue culture system (Zuniga et al., 2007). Since G8R is a late gene transcriptional activator, it is predictable that late gene expression is blocked. Moreover, in the presence of IFN treatment, in the E3L deletion mutant infections, the G8R protein is absent. Apart from antagonizing PKR induced eIF2α phosphorylation, it is also possible that E3L can directly regulate the expression of other VV genes involved in viral transcription and/or translation. In fact, a recent study that describes the association of VV transcription and translation with cytoplasmic DNA factories illustrates the relocation of E3L to these DNA factories at times post-infection at which intermediate and late viral gene expression would occur (Katsafanas and Moss, 2007).

In this report, we demonstrate that VV E3L is a potent inhibitor of both type I and type II human IFNs. We found that the main mechanism by which VV E3L inhibits human IFN activity in Huh7 cells is by modulation of the PKR pathway. Of importance, VV E3L and its homologs in other human pathogenic orthopoxviruses are highly conserved. Thus, the IFN antagonizing activity of VV E3L described in this report is significant to the understanding of human orthopoxvirus immunopathogenesis. Collectively, this report highlights an excellent human cell line system in which to study the inhibition activity of a strong IFN antagonist, VV E3L, against all human type I and type II IFN species. This system has the potential in studying other viral IFN antagonistic proteins whose means of innate evasion is primarily by modulation of the PKR pathway.

## Materials and methods

### *Cell culture and viruses*

Baby hamster Kidney (BHK21), HeLa-S3, HepG2, SW13, MRC5, AD293 (ATCC) and Human Hepatoma (Huh7) (provided by Dr. G.Y. Minuk, University of Manitoba) cell lines were maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum and antibiotics (Invitrogen). The virus used in this study is vaccinia virus Copenhagen strain (provided by Dr. G. McFadden, University of Florida).

### *Human interferons*

Type I IFNs (12 IFN-α subtypes, IFN-β1a, IFN-ω), leukocyte IFN (a mixture of IFN-α subtypes and IFN-ω) and type II IFN (IFN-γ) were purchased from PBL Biomedical Laboratories.

### *Construction of VV Copenhagen E3L deletion (pΔE3L/EGFP) and E3L deletion-Revertant (pΔE3L/EGFP-Rev) recombinant vectors*

For homologous recombination, the left and right flanking regions of the *E3L* gene were PCR amplified using VV Copenhagen strain genomic DNA as the template. The left flanking region was synthesized with primer pair of E3L_FLF (ATTA***CTCGAG***TGATGTTCATATATTGGTT-***TTGTCCCGGGTTGAAGCTTACTACATATGAGAATGCTAGCTAGAT***TCT-GATTCTAGTTATCAATAACA), while the right flanking region was synthesized with primer pair of E3L_FRF (***ATCTAGCTAGCATTCTCA-ACAA***CCAGCAATAAAACTGAACCTACT) and E3L_FRR (TGGC***GAGCTC***T-CTAA). The complementary sequences representing multiple cloning sites are shown in bold and italics. The left and right flanking sequences were ligated by a second round PCR with primers FLF and FRR and were inserted into cloning vector pBS-KS+ (Stratagene) between the XhoI and SacI sites, resulting in plasmid pΔE3L. Sequence fidelity was confirmed. The *gpt* gene driven by an early and late promoter, p7.5, was subcloned into pΔE3L at the SmaI site. A cassette containing *EGFP* gene, driven by an early and late promoter, was cloned between NheI and NdeI sites, yielding VV Copenhagen *E3L* deletion vector.

To construct the revertant control vector, the *E3L* gene and its authentic promoter region was PCR amplified from VV Copenhagen strain genomic DNA with primer pairs E3L_N-XhoI 5′-ATTACTCGA-GATTTCGCAATCTTAATGTTACAACG-3′ and E3L_C-SmaI 5′-ATAACCC-GGGTCAGAATCTAATGATGACGTAACCAA-3′. The *E3L* gene was cloned into pΔE3L/EGFP, resulting in plasmid pΔE3L-Rev.

### *Generation of VV Copenhagen E3L deletion and control ΔE3L-Revertant recombinant viruses*

5 μg of plasmid DNA pΔE3L/EGFP (for generation of the E3L deletion mutant, Cop-ΔE3L) was transfected into BHK21 cells infected with Copenhagen strain wild-type using Effectene Transfection Reagents according to the manufacturer's instructions (Qiagen). Recombinant poxvirus was grown in selective media containing mycophenolic acid (Falkner and Moss, 1988), and purified after 4 rounds of plaque purification. For generation of the revertant control virus, 5 μg of plasmid DNA pΔE3L-Rev was transfected into BHK21 cells infected with the E3L deletion mutant virus. The purity of the Cop-ΔE3L mutant and revertant control Cop-ΔE3L-Rev viruses was confirmed by PCR with primer pair flanking the *E3L* ORF, forward primer 5′-TGATAAAGTAGGTT-CAGTTTTATTGCTGGTTGT-3′ and reverse primer 5′-TGTTATTGATAACTA-GAATCAGAATCTA-3′. Adjacent ORFs were amplified by PCR with the following primer pairs: *E2L* forward primer 5′-TGGATTCTGTCCAATGAT-GATGAAACG -3′ and reverse primer 5′-TCTTCCCTCTATCATGTTCAC-TACTGG-3′; *E4L* forward primer 5′-ATACATTAGTAGTTACTCATCCAA-3′ and reverse primer 5′-ATCATCCTCTGGTGGTTCGTCGTT-3′.

### *Interferon sensitivity assays*

Huh7 cell monolayers were grown in 12-well culture plates to 80% confluency and subsequently treated overnight at 37 °C with varying concentrations of human IFNs (12 Human IFN-α subtypes, IFNs-β, ω, and γ) at 0, 2, 20, 200, or 2000 U/ml. Cell monolayers were infected at a multiplicity of infection (MOI) of 1 with either Cop-ΔE3L-Rev or Cop-ΔE3L recombinant viruses and incubated at 37 °C. One hour post-infection (hpi), total virus and media containing IFN were aspirated, and replaced with fresh media containing the same concentration of IFN, and incubated for an additional 24 h at 37 °C. Control wells contained growth media without IFN.

### *Plaque assays*

Cop-ΔE3L-Rev and Cop-ΔE3L viruses were collected 24 hpi and serial dilutions of each were used to infect 100% confluent BHK21 cell monolayers. To calculate virus titre (pfu/ml), plaques were enumerated 24 hpi under UV. Titrations were performed in triplicate.

### *Western blot*

Western blots were performed to examine the profile of signaling proteins in Huh7 cells primed with type I and type II IFNs. In brief,

Huh7 cells were treated overnight at 37 °C with 1000 U/ml of either leukocyte IFN, IFNs-β, ω, and γ, and infected at a MOI of 5 with either Cop-ΔE3L-Rev or Cop-ΔE3L. To inhibit cellular phosphatase activity, Phosphatase Inhibitor Cocktail I (Sigma-Aldrich) was added to all wells 6 hpi, prior to cell lysis in PBS containing SDS. Proteins were separated by electrophoresis on 4–12% polyacrylamide gels (Bio-Rad) in 1× XT Mops Running Buffer (Bio-Rad) at 150 V for 1 h, transferred onto H-bond nitrocellulose membranes (Amersham Biosciences) at 150 mA for 1 h, and the membranes were incubated with polyclonal antibodies against STAT1 (Upstate) and phospho-STAT1 (Y701) (Upstate), phospho-STAT1 (S727) (Cell Signaling Technology), PKR and phospho-PKR (T446) (Epitomics), and eIF2α and phospho-eIF2α (S51) (Biosource) in 5% skim milk in TBST (1× Tris–borate sulphate and 0.2% Tween 20). Polyclonal antibodies for VV D12L and VV G8R were prepared against the peptide sequences RERDAIKSNNHLTE and TPGNTDAFSREYSM, respectively (Genscript). A monoclonal antibody against human RNaseL (Cell Signaling Technology) was also used in this study. Proteins were detected using chemiluminescence-based Western Lightning Reagents (PerkinElmer LAS, Inc).

*RNA gel for RNaseL activity*

Huh7 cells were treated with 1000 U/ml of leukocyte IFN, IFN-β, IFN-ω, and IFN-γ, and infected at a MOI of 5 with either Cop-ΔE3L-Rev or Cop-ΔE3L. Total RNA was extracted 6 and 12 hpi using the RNeasy Mini Kit (Qiagen) according to the manufacturer's protocol. Samples were prepared according to the NorthernMax Protocol (Ambion, Inc.) and loaded onto 1% agarose-LE (Ambion, Inc.), electrophoresed at 100 V for 1 h, and visualized under UV light.

*PKR and MxA RNA silencing*

Huh7 cells were seeded at $3\times10^5$ cells per well in 6-well culture plates and transfected with 100 nM of small interfering RNA (siRNA) specific to human *PKR* 5′-P-ACUUUGUCUAGUUUCUCGCUU, and *MxA* 5′-P-UUCGUCUUCCGGUAUGUCGUU (Dharmacon RNA Technologies) and *Silencer* Cy3-Labeled Negative Control #1 (Catalog #4621, Ambion, Inc.) using Hiperfect Transfection Reagent (Qiagen) in DMEM and incubated at 37 °C. 48 h later, cells were treated with 100 U/ml of leukocyte IFN, IFN-β, IFN-ω, and IFN-γ overnight, then infected at a MOI of 1 with Cop-ΔE3L. Virus titres were determined by plaque assays. *PKR* silencing was confirmed by Western blot with an antibody against PKR (Cell Signaling Technology), and the suppression of *MxA* was confirmed by RT-PCR.

*Reverse transcription polymerase chain reaction (RT-PCR)*

Reverse transcription was performed using Advantage RT-for-PCR Kit (Clontech) according to the manufacturer's instructions. Negative cDNA controls were prepared under identical reaction conditions without reverse transcriptase. PCR was performed with *MxA* forward primer 5′-AAGGTCAGTTACCAGGACTACGAGA-3′ and reverse primer 5′-ACAATCATGTAACCCTTCTTCAGGT-3′; human *RNaseL* forward primer 5′-TATGGCTTCACAGCCTTCATGGAA-3′ and reverse primer 5′-ACAATCTGTACTGGCTCCACGTTT-3′; human *GAPDH* forward primer 5′-AAGGTGAAGGTCGGAGTCAACGGA-3′ and reverse primer 5′-TTACTCCTTGGAGGCCATGT-3′; *vvD12L* forward primer 5′-ATGGATGAAATTGTAAAAAATATCCGGGA-3′ and reverse primer 5′-TCACAGCAGTAGTTTAACTAGTCT-3′; *vvG8R* forward primer 5′-AATGTAGACTCGACGGATGAGTTA-3′ and reverse primer 5′-TCGTCATTATCCATTACGATTCTAGTT-3′.

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